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A Specific Detection Method for Multiple Forms of Cystine Aminopeptidase (Oxytocinase-Isoenzymes) after Polyacrylamide Gel Electrophoresis

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Summary: A simple and specific detection method for multiple forms of cystine aminopeptidase (oxytocinase-isoenzymes) in pregnancy serum is described using S-benzyl-*L*-cysteine-*p*-nitroanilide as substrate. The relative distribution of both cystine aminopeptidase isoenzymes at different stages of pregnancy is given.

Eine spezifische Methode zum Nachweis der multiplen Formen von Cystin-Aminopeptidase (Oxytocinase-Isoenzyme) nach Polyacrylamidgel-Elektrophorese

Zusammenfassung: Es wird eine einfache und spezifische Methode zum Nachweis der multiplen Formen von Cystin-Aminopeptidase (Oxytocinase-Isoenzyme) im Serum Schwangerer unter Verwendung von S-Benzyl-*L*-cystein-*p*-nitroanilid als Substrat beschrieben. Die relative Verteilung der beiden Cystin-Aminopeptidase-Isoenzyme in verschiedenen Perioden der Schwangerschaft wird mitgeteilt.

Introduction

The detection methods for oxytocinase-isoenzymes (cystine aminopeptidase 1 and 2) (EC 3.4.11.3) in pregnancy serum after polyacrylamide gel electrophoresis using *L*-leucine-β-naphthylamide as substrate are non-specific (1, 2, 3). Both cystine aminopeptidase 1 and 2 and leucine aminopeptidase activity (EC 3.4.11.2) are always detected. Using *L*-cystine-di-β-naphthylamide as substrate, only the cystine aminopeptidase isoenzymes are demonstrated (4), but both bands on the gel are too faint for scanning. Moreover, β-naphthylamides are carcinogenic.

L-Cystine-bis-*p*-nitroanilide and S-benzyl-*L*-cysteine-*p*-nitroanilide are split only by oxytocinase, but in the assay of oxytocinase the first substrate is less sensitive than S-benzyl-*L*-cysteine-*p*-nitroanilide (5). We therefore propose a new specific detection method for cystine aminopeptidase 1 and 2, using a substrate that does not have these disadvantages: S-benzyl-*L*-cysteine-*p*-nitroanilide (6). After electrophoresis the liberated *p*-nitroaniline is diazotized during the enzymatic reaction, then coupled with N-(1-naphthyl)-ethylene-diamine to yield pink coloured bands (7).

Materials and Methods

The separation of cystine aminopeptidase isoenzymes is based on the method of l.c. (1).

To achieve good and reproducible separation of both isoenzymes we have adopted a 75 g/l acrylamide concentration in the separation gel and 0.078 mol/l borate buffer pH 8.6.

Polyacrylamide gel electrophoresis was carried out with a Canalco Research Disc Electrophoresis Equipment model 1200 and a Electrophoresis Constant Rate Source model 100 for 1.5 h at 0° C (5 mA/gel). The detection of the isoenzymes is performed as follows.

Reagents for the detection of cystine aminopeptidase isoenzymes

1. Dissolve 26 mg S-benzyl-*L*-cysteine-*p*-nitroanilide (Boehringer Mannheim or British Drug House) in 20 ml 2-methoxyethanol (4 mmol/l). Store in a brown bottle at 4°C.
2. Citrate-phosphate buffer pH 6.0. Mix 36.8 ml of 0.1 mol/l citric acid with 63.2 ml of 0.2 mol/l Na_2HPO_4-solution. Store at 4°C.
3. Dissolve 50 mg $NaNO_2$ and 10 mg N-(1-naphthyl)-ethylene-diamine · 2 HCl (J. T. Baker Chemicals) in 50 ml reagent 2 at 37° C. Prepare before use.
4. Incubation reagent. Add reagent 1 to reagent 3 in a ratio 30 ml + 70 ml.
5. Dissolve 125 g trichloroacetic acid in 1 l water.

Proposed detection-method

After electrophoresis incubate the gels in the incubation reagent for 1 h at 37°C. Wash the gels 1 min with water to remove excess $NaNO_2$ and substrate.

Incubate the gels 1 min in reagent 5. Scanning of the gels was performed with a densitometer Kipp Skalar Densiscan KS 3 filter 530 nm and a XY-Recorder BD 22.

The total serum oxytocinase catalytic concentration was determined kinetically (5).

Results and Discussion

After incubating the gels in the incubation reagent two strong pink bands were obtained (Fig. 1). The intensity of the pink bands became maximal 5–6 min after treatment with trichloroacetic acid and remains constant for 1 h. In sera with low levels of total oxytocinase catalytic concentration (12–19 U/l) the first isoenzyme to appear, i. e. cystine aminopeptidase 1, can be easily detected. In a serum with a total oxytocinase catalytic concentration of 37 U/l both the cystine aminopeptidase isoenzymes (CAP_1 and CAP_2) can be readily demonstrated (CAP_1 61% and CAP_2 39% of the total activity). In late pregnancy sera (total oxytocinase catalytic concentration 115–144 U/l) the activity of cystine aminopeptidase 2 is in general greater than the activity of cystine aminopeptidase 1: CAP_1 37–44% and CAP_2 56–63% of the total activity (Fig. 2).

From the literature (1, 2, 3) it is not clear which isoenzyme appears first in the serum during pregnancy. *Kleiner & Brouet-Yager* (4) stated that cystine aminopeptidase 2 is the first isoenzyme to appear. We could not confirm this. Preliminary results with our detection method show that cystine aminopeptidase 1 is the earliest demonstrable isoenzyme.

The relative electrophoretic mobilities of the cystine aminopeptidase isoenzymes 1 and 2 are: 0.60 ± 0.01 and 0.26 ± 0.02 ($\bar{x}$ ± SD, N = 10) respectively, using bromophenol blue as tracking dye.

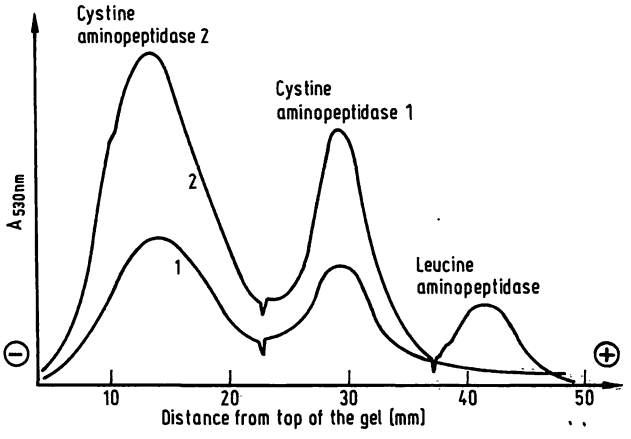

Fig. 1. Detection of cystine aminopeptidase isoenzymes 1 and 2 (CAP_1 and CAP_2).
1. Our detection method, substrate S-benzyl-*L*-cysteine-*p*-nitroanilide (4 mmol/l).
2. Detection method according to l. c. (1), substrate *L*-leucine-β-naphthylamide (4 mmol/l). Total serum oxytocinase catalytic concentration 116 U/l.

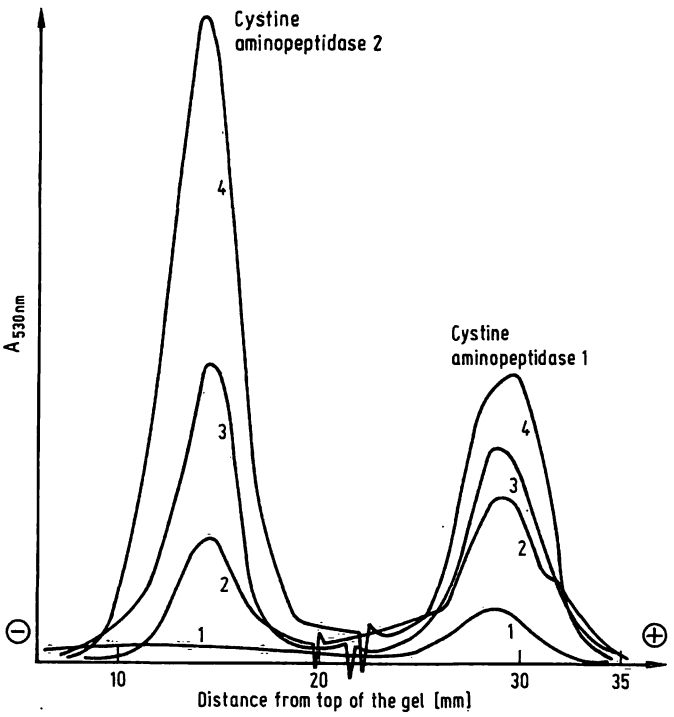

Fig. 2. Relative distribution of the cystine aminopeptidase isoenzymes 1 and 2 (CAP_1 and CAP_2) in sera of different pregnant women.
1. Total serum oxytocinase catalytic concentration 14 U/l; CAP_1 100%, CAP_2 0%.
2. Total serum oxytocinase catalytic concentration 37 U/l; CAP_1 61%, CAP_2 39%.
3. Total serum oxytocinase catalytic concentration 76 U/l; CAP_1 49%, CAP_2 51%.
4. Total serum oxytocinase catalytic concentration 144 U/l; CAP_1 37%, CAP_2 63%.
(Sensitivity in Y-direction is greater than in fig. 1).

In contrast with *Tovey* et al (8) we could not observe any influence on the intensity of the CAP_1 and CAP_2 bands, when varying the 2-methoxyethanol concentration from 100 to 300 ml/l in the incubation-reagent. We used a 300 ml/l 2-methoxyethanol concentration, because of the solubility of S-benzyl-*L*-cysteine-*p*-nitroanilide. The concentrations of $NaNO_2$ and N-(1-naphthyl)-ethylenediamine given by (7) were too high, resulting in a turbid incubation reagent. We decreased these concentrations to give final concentrations of 10 mmol/l and 0.5 mmol/l for $NaNO_2$ and N-(1-naphthyl)-ethylene-diamine respectively, which gave a clear incubation reagent when dissolved at 37°C.

Further studies on the relative distibution of the cystine aminopeptidase isoenzymes in normal and high risk pregnancy sera are in progress.

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References

1. Kleiner, H. & Schram, E. (1966), Clin. Chim. Acta *14*, 377–385.
2. Oya, M., Yoshino, M. & Asano, M. (1974), Experientia *30*, 985–986.
3. Mizutani, S., Yoshino, M. & Oya, M. (1976), Clin. Biochem. *9*, 16–18.
4. Kleiner, H. & Brouet-Yager, M. (1972), Clin. Chim. Acta *40*, 177–180.
5. Van Buul, T. & van Oudheusden, A. P. M. (1974), Clin. Chim. Acta *54*, 263–268.
6. Van Oudheusden, A. P. M. (1972), J. Clin. Chem. Clin. Biochem. *10*, 345–346.
7. Dahlmann, B. & Jany, K. D. (1975), J. Chromatogr. *10*, 174–177.
8. Tovey, J. E., Dawson, P. J. G. & Fellowes, K. P. (1973), Clin. Chem. *19*, 756–761.

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